Supplemental Material:

XANES: Scanning transmission X-ray microscopy and spectroscopy were performed at beam line (BL) 5.3.2 at the Advanced Light Source (ALS), Lawrence Berkeley Laboratory (Kilcoyne et al., 2003). BL5.3.2 employs a bending magnet to provide photons in the 250–700 eV energy range. Energy selection is performed with a low-dispersion spherical grating monochromator (E/ΔE = 5000) calibration with CO_2 and N_2 gas. Fresnel zone plate optics provide a focused beam on the order of 25 nm. XANES spectra were acquired using a multi-spectral imaging method (Jacobsen et al., 2000). The energy step size (ΔE) in the fine structure regions was 0.1 eV, in the less featured pre-edge and post edge regions, energy steps of 1–2 eV is sufficient for spectral resolution.

In general, absorption at the lowest energies (~ 285.0 eV) is well described by photo-excitation of carbon 1s electrons to low energy, unoccupied, π* orbitals of alkenyl and aromatic species (C and H substituted). Carbon substitution with more electronegative atoms, e.g., N and O, results in shifts to higher excitation energies, e.g., 288.2 and 288.5 eV in the case of carbonyl 1s- π* transitions of amides and carboxyls, respectively. Saturated carbon, i.e., methyl and methylene, also exhibit relatively intense absorption in the near edge region around 287.5 EV commonly identified as a 1s-3p/σ* transition. The energy of the 1s-3p/σ * transition is also affected by the electronegativity of neighboring atoms, e.g., N and O, which leads to a shift of the 1s-3p/σ* transition to higher energies, e.g., from ~ 287.5 eV for simple hydrocarbons up to ~ 289.5 eV for

alcohols. Unsaturated nitrogen species are readily distinguished from each other as there are significant shifts, ~3.5eV, in energy from imine and nitro N 1s-π* transitions. Similarly, the 1s-3p/σ* transitions of Nitro N are shifted from amine, amidyl and pyrrolic NH. O-XANES spectra provide information regarding the proportion of carbonyl vs alcohol and/or ether. In general carboxyl, ketone, and aldehdye carbonyls exhibit an intense 1s-π* transition at 531.1 eV, amide and peptide carbonyl 1s-π* transitions are shifted to 532.2 eV. The alcohol and ether O 1s-3p/σ* lies at ~ 535 eV and is typically observed as a shoulder on the O 1s absorption edge and associated 1s-σ* virtual state absorption.

Oxygen XANES:

Oxygen XANES provides information on the fraction of oxygen that exists as carbonyl, e.g., ketone, carboxyl, amidyl, and in sp^3 bonded configurations, e.g., alcohol and ether. The O-XANES spectrum of chitin is relatively simple (Fig. s 1a & b), with a small peak at 532.2 eV corresponding to amidyl carbonyl 1s-π* transition. Intense absorption associated with the abundant secondary alcohols, i.e., the OH 1s-3p/σ* transition, exists at ~ 535 eV, but is difficult to resolve from the intense virtual state, O "1s-σ*", absorption that overlaps the ionization edge. The O-XANES spectrum of modern scorpion cuticle reveals a considerably greater proportional intensity of the amidyl carbonyl 1s-π* region (at ~ 532 eV) relative to alcohol or ether when compared to chitin's O-XANES spectrum, consistent with the considerable concentration of protein in addition to chitin in scorpion cuticle, and a lower relative abundance of glucosyl hydroxyls. In the O-XANES spectrum of modern scorpion there is also a slight shift in the carbonyl 1s-π* transition towards 531 eV, indicating the presence of either ketone or

carboxyl moieties. The O-XANES spectrum of fossil scorpion cuticle reveals somewhat smaller proportion of oxygen in the form of carbonyl 1s-π* transition absorption relative to alcohol and/or ether when compared to modern scorpion. The shift in energy of the carbonyl 1s-π* transition from 532.2 eV to 531.1 eV indicates that the dominant carbonyl species in the fossil cuticle are no longer amide or peptide, but are now ketone and/or carboxyl, consistent with what is observed in the C-XANES spectrum (Fig. s 1a & b). Note that some amidyl carbonyl still exists as evidenced by the shoulder at 532.2 eV (Fig. s 1b). The carbonyl 1s-π* absorption band in the O-XANES spectrum of the eurypterid fossil is broad and lies between the characteristic absorption for amide (532.2 eV) and ketone/carboxyl (531.1 eV) indicating the presence of both types of carbonyls being present in the eurypterid fossil organic macromolecule. It is noteworthy that the relative abundance of carbonyl relative to total oxygen is lower than the modern and fossil scorpion. The remainder of the oxygen exists as either alcohol or ether.

Elemental Ratios derived from C-, N-, and O-XANES spectra:

One can fit the C-, N-, and O-absorption edge intensity using published atomic absorption cross section data to obtain quantitative O/C and N/C data (e.g., Cody et al. 2008 and references therein). In the case of chitin one obtains from these X-ray absorption data (Fig. s2) O/C = 0.78 and N/C = 0.13 which compares well with the expected values of O/C = 0.75 and N/C = 0.125, or $C_{100}N_{12.5}O_{75}$. Fitting the combined C-, N-, and O-XANES edge intensities of the modern scorpion cuticle (Fig. s3) yields an O/C of 0.33 and N/C of 23, or $C_{100}N_{23}O_{33}$, consistent with a mixture of chitin and protein. Fitting the C-, N-, and O-XANES ionization edge data (Fig. s4) of the fossil scorpion

cuticle yields values of N/C = 0.10 and O/C = 0.29, or $C_{100}N_{10}O_{29}$. Thus, while the oxygen content of the fossil scorpion cuticle remains nearly the same as the modern scorpion, the nitrogen content has dropped by slightly more than half. Finally, fitting the C-, N-, and O-XANES data of the eurypterid fossil cuticle (Fig. s5) yields the elemental composition N/C = 0.08 and O/C = 0.17, or $C_{100}N_{8}O_{17}$.

Estimation of the molecular percentage of carbon in chitin and protein in modern scorpion cuticle: Whereas the elemental composition of chitin is easily determined as chitin is a highly regular polymer of poly-N-acetyl-D-glucosamine ($C_{100}N_{12.5}O_{75}$) the structural proteins in arthropod cuticles are composed of various amino acids. Højrup et al. (1986) determined the primary structure of structural protein from a migratory locus cuticle. As might be expected for a structural protein, relatively few amino acids are used. Predominantly the protein is composed of Alanine, valine, proline, tyrosine, and glycine, when normalized to 100 % correspond to 52 %, 14 %, 14 %, 12 %, and 8 %, respectively. Computing an elemental formula for this protein yields an elemental formula for the protein of $C_{100}N_{28.2}O_{29.5}$. Having determined the elemental formula for the modern scorpion as being $C_{100}N_{23}O_{33}$, one can establish the distribution of chitin and protein in the modern cuticle to be 33 % chitin-67 % protein, using N abundance as the constraining value. This compares very well with solid-state ^{13}C NMR spectra of the same material (figure s6) from which it can be determined that carbon exists as 34 % chitin and 66 % protein.

Inner-shell N absorption spectrum of Nitro benzene: Direct bonding of two electron withdrawing oxygen atoms to nitrogen in the nitro group shifts the binding energy of the N (1s) electrons and shifts the N 1s-π^* transition to a higher energy then all other organo nitrogen functional groups. In Figure s7 we reproduce the inner shell N 1s spectrum of nitrobenzene as published by Turci et al. (1996) along with the N XANES spectra of modern and ancient scorpion cuticle. The intense peak observed in the N XANES spectrum of ancient scorpion is most likely indicative of organo nitro ($R\text{-}NO_2$) functionality.

Compositional Mapping via STXM Imaging: One of the most powerful aspects of Scanning Transmission X-ray Microscopy (STXM) is the capability of obtaining images wherein contrast is derived from spatial variation in chemical composition. Compositional mapping is performed by acquiring a pair of images, one at an energy corresponding to the functional group of interest (I) the other acquired at an energy just below the XANES features (this image provides an effective I_o), the compositional map is derived by presenting the resultant image of $-\log(I/I_o)$. Variations in the targeted functional group are revealed where lighter regions are richer in concentration, darker regions are depleted. For example, in Fig. s8A, a compositional map of a cross section through modern scorpion cutitcle is presented wherein the variations in amide nitrogen is revealed by presenting the $-\log(I_{401.3}/I_{380})$. Clearly defined lamina are observed, where the outer region of the cuticle (top) is clearly depleted in amide nitrogen. Within the interior brigher bands of amide/peptide nitrogen are observed. In fig. s8b, a compositional map of aliphatic carbon [$-\log(I_{287.5}/I_{282})$] highlights the enrichment of

aliphatic carbon in outer region of the cuticle as well as within irregular bands in the inner region of the cuticle. As protein will have proportionally more aliphatic carbon than chitin, it is reasonable to presume that the irregular bands rich in both aliphatic carbon (fig s6b) and amide/peptide nitrogen (fig. s6a) are proportionally more enriched in protein. To test this, we acquire a map of amide/pepide carbon [$-\log(I_{288.2}/I_{280})$] shown in fig. s6C where is clear that peptide carbon is concentrated in the irregular lamina in the interior of the cuticle. Using the approach of compositional mapping available via STXM, only three maps are necessary to reveal the spatial variation in the composition of major biomolecular constituents of arthopod cuticle, i.e., chitin, protein, and waxes.

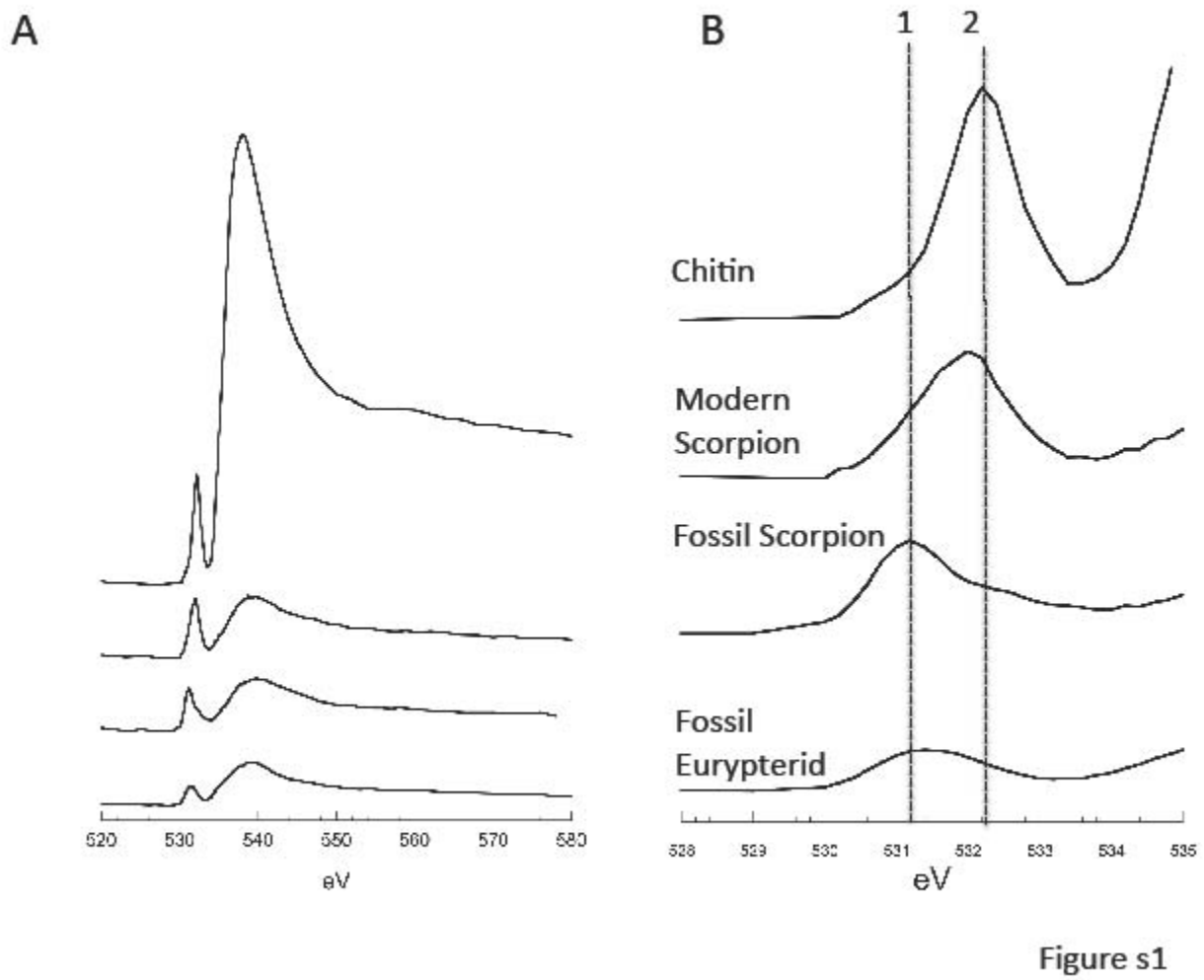


Figure DR1: A, O-XANES spectra of chitin (top), modern scorpion (mid-top), fossil scorpion cuticle (mid-bottom), and fossil eurypterid cuticle (bottom). Variation in the intensity of the carbonyl 1s-π^* intensity (at ~ 531.5 eV) with intensity at ~ 540 eV reflects variation in C=O vs C-O. **B**, expanded view of the carbonyl 1s-π^* transition, where absorption at **1** (531.2 eV) corresponds to carbonyl in ketone and carboxyl and absorption at **2** (532.2 eV) corresponds to carbonyl in amide/peptide.

Figures DR2-DR5: Full edge fits of the C-, N-, and O-XANES spectra to atomic absorption cross-section from which atomic N/C and O/C are obtained.

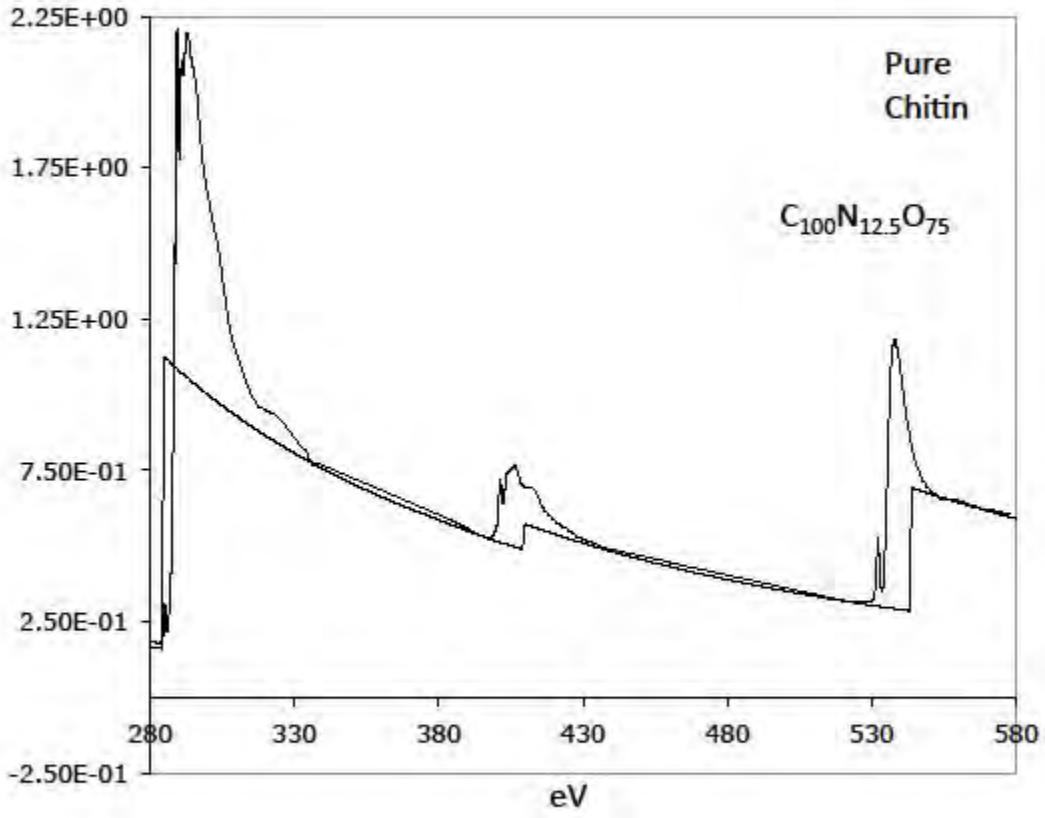
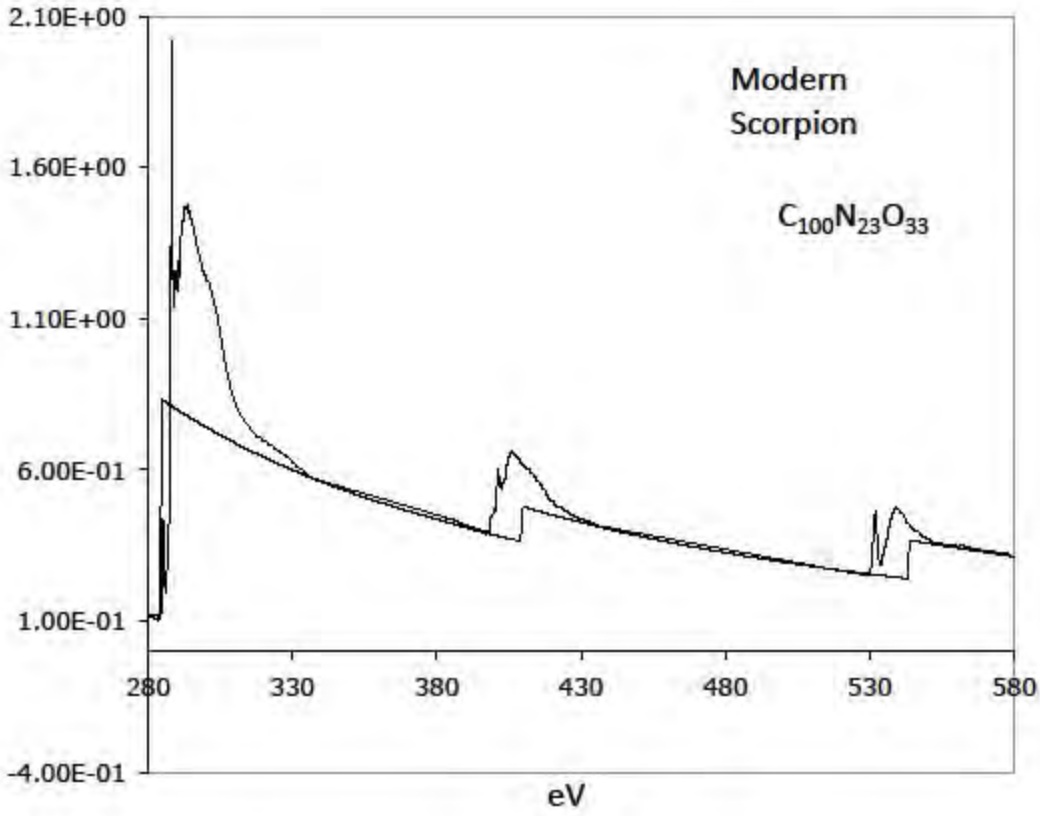


Figure s2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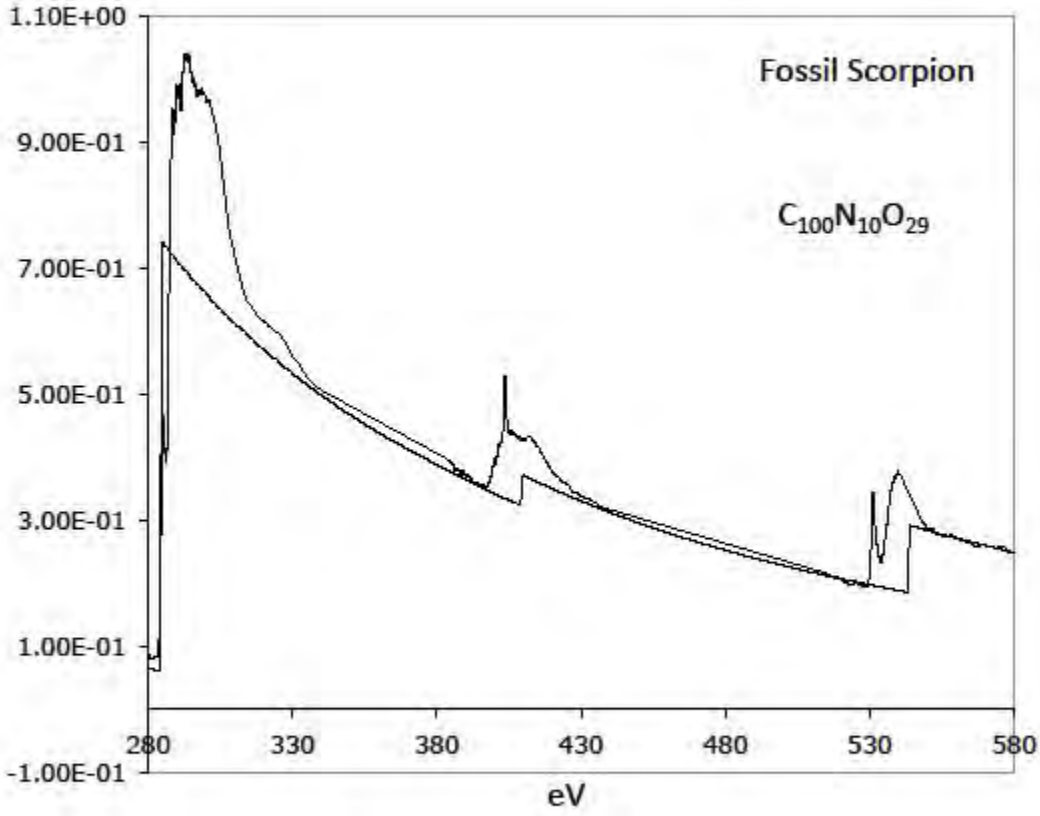
1.60E+00
1.10E+00
6.00E-01
1.00E-01
-4.00E-01
280
330
380
430
480
530
580
eV
Modern
Scorpion
$C_{100}N_{23}O_{33}$

Figure s3


Fossil Scorpion
$C_{100}N_{10}O_{29}$
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9.00E-01
7.00E-01
5.00E-01
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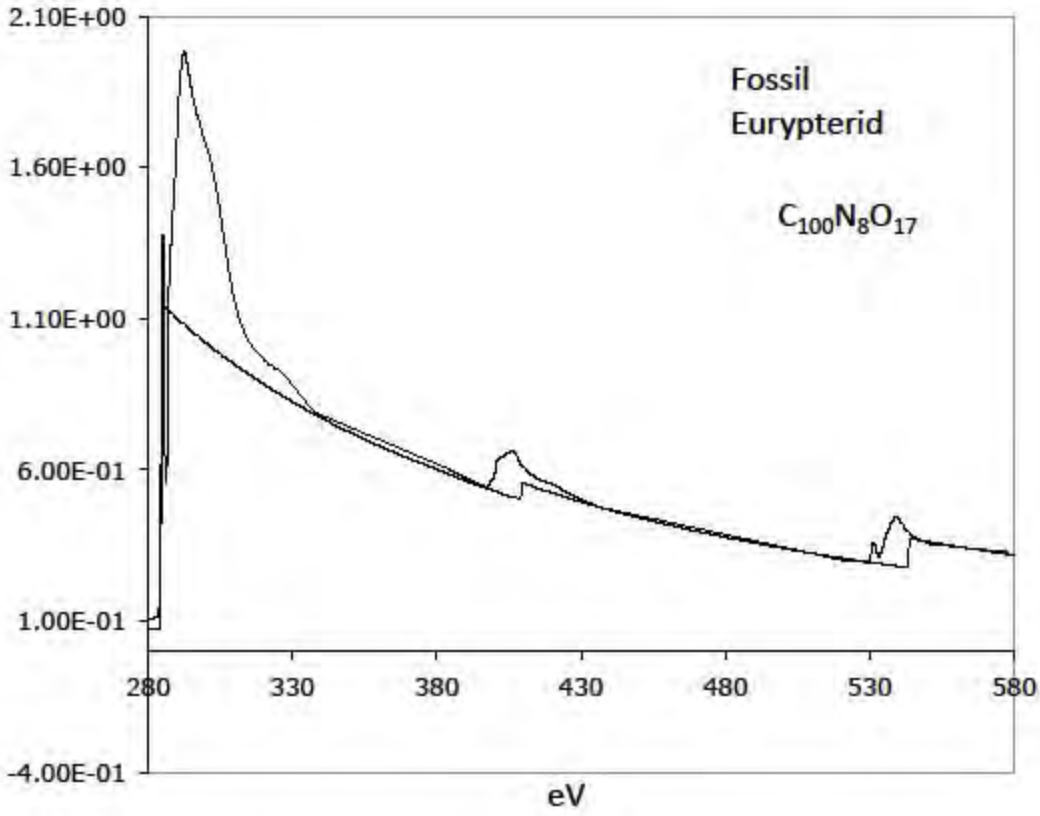
1.00E-01
-1.00E-01
280
330
380
430
480
530
580
eV

Figure s4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-4.00E-01
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580
eV
Fossil
Eurypterid
$C_{100}N_8O_{17}$

Figure s5

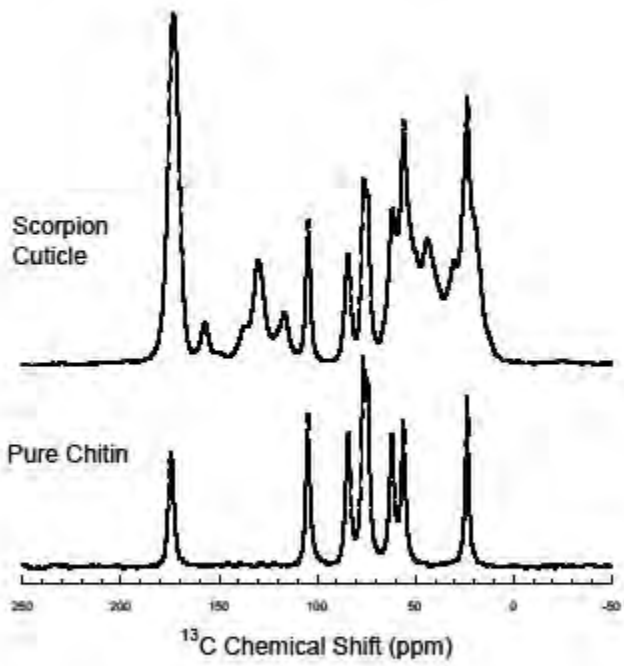

Figure s6

Figure DR6: ^{13}C solid-state NMR spectrum of modern scorpion cuticle and chitin. Integration of the spectrum of the modern scorpion indicates that the cuticle is composed of 36 % chitin and 64 % protein.

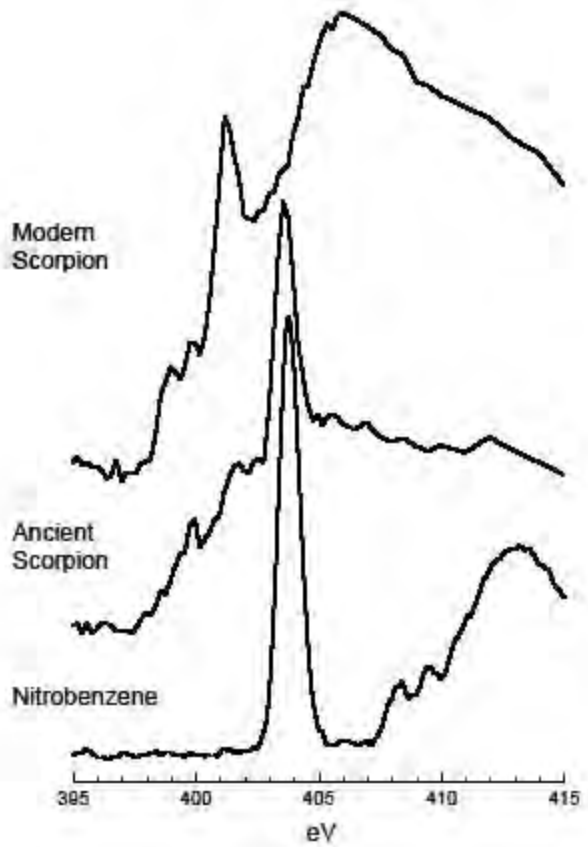

Figure s7

Figure DR7: N inner shell (1s) spectra of modern scorpion cuticle (top), fossil scorpion cuticle (middle), and pure nitrobenzene (bottom). The modern scorpion N-XANES spectrum reveals the characteristic 401.3 eV peak consistent with amidyl nitrogen. The ancient scorpion cuticle N-XANES spectrum exhibits weak intensity at 401.3 eV and a strong absorption feature at 403.65 eV. This sharp high-energy feature likely indicates the presence of organo nitro groups. The nitrogen inner shell (1s) spectrum of nitrobenzene exhibits a sharp 1s-π^* transition at 403.7 eV.

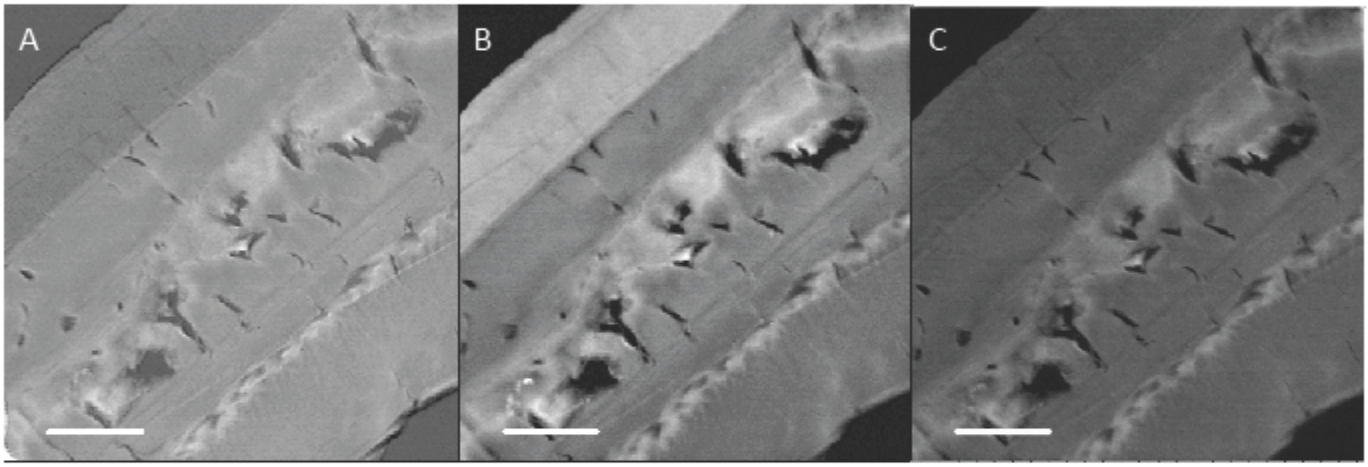

Figure s8

Figure DR8: Compositional maps derived from –log absorption ratio contrast, a) variation in the spatial distribution of amide/peptide nitrogen, b) variation in the abundance of aliphatic carbon, and c) variation in the abundance of amide/peptide carbon.